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Research on the development of a short term standard toxicity test with *Artemia* nauplii

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Abstract

Standardization of toxicity tests on aquatic organisms to detect the impact of chemicals on freshwater or marine ecosystems is an urgent necessity.

From the variety of methods, criteria, and test species proposed, simple standardized tests for algae, crustaceans, and fish are now close to being adopted at the international level for the freshwater environment. Despite the same urgency, very little has been achieved for the marine environment.

Artemia is an extremely suitable test-species since, contrary to all other organisms, it does not require continuous maintenance of stock cultures. Unfortunately none of the *Artemia* toxicity tests that have been worked out so far can be adopted as such as a representative standard test, because they attribute too little importance to parameters, which influence the results and the repeatability.

Starting from the pertinent literature information on the use of *Artemia* as a test species for toxicity studies and from literature data on the factors influencing the hatching and molting of brine shrimp, we studied the parameters of importance for a routine short-term toxicity test with an acceptable reproducibility, which are :

- selection of the instar stage of the nauplii
- determination of the test's duration
- sensitivity of early *versus* late hatching nauplii
- influence of the storage conditions of the cysts
- sensitivity of different geographical strains and batches of *Artemia*
- selection of a standard reference toxicant
- determination of the accuracy and repeatability of the test

Introduction

The increasing concern of reducing the adverse effects of chemicals on the ecosystems has led to the development of a large number of ecotoxicological tests for the predictive assessment of the potential effects of pollutants on the biota.

In view of the regulatory measures which are now taken at the national as well as at the international level, it is obvious that standardization of the methods which will be witheld or recommended at the different levels of complexity of testing is highly desirable.

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This is especially the case for routine tests, which must be as reproducible, reliable, and simple as possible. This automically means that the tests should not require sophisticated equipment nor specialized personnel.

Among the numerous species that have been used for toxicity studies in the marine environment (Wisely and Blick, 1967 ; Connor, 1972 ; Standard Methods, 1975 ; Stephan, 1975), the brine shrimp appears to be a very suitable candidate to develop a standard bioassay for worldwide utilization. Dry *Artemia* cysts are indeed available year-round and can be shipped very easily in small packages to any point of the world. This means that the test can be carried out wherever and whenever, starting from biological material stored "on the shelf". As a result the cumbersome and expensive maintenance of stock cultures is completely eliminated.

These advantages of *Artemia* have led to a marked increase of the use of this organism for bioassay purposes during the past two decades. Unfortunately, a study of the literature reveals, however, that there is no uniformity in the methodologies nor in the criteria used to assess the dose-effect relationship.

Departing from the urgent necessity of standardizing a few bioassay methodologies representative for the marine environment, and considering the advantages inherent to the use of *Artemia* as a suitable candidate, we studied a number of parameters of importance to develop a routine short term toxicity test with this particular species. A thorough review of the existing literature dealing with the use of *Artemia* in toxicity testing was at the basis of our studies.

Literature review

The dose-effect criteria used to assess toxicity on brine shrimp can be classified in three categories according to the life-stage of the test-species :

a) cysts
b) nauplii
c) adults

The hatching process as criterion

As far as we know, Delcambe (1955) was the first scientist who used the hatching success as a bioassay criterion. His method has been followed by that of Pham-Huu-Chanh and Mamy (1963). De Brabander and Vandeputte (1967) took several criteria into consideration : the percentage of unhatched cysts, that of the cysts in the breaking stage, and the percentage of free-swimming nauplii. Jensen (1975) also proposed the use of the hatching rate as a standard bioassay technique. His method, slightly modified, has been used by Jörgensen and Jensen (1977, 1978). Finally, Saliba and Krzyz (1976a) studied the influence of heavy metals on the hatching of *Artemia* cysts.

An intercomparison of the dose-effect relationship obtained by different authors using the hatchability criterion is hardly possible. Indeed, the hatching conditions used varied widely. Temperature for example varied from 19 up to 28 °C and the duration of the test from 48 hr up to 5 days. Moreover, in most cases abiotic factors such as pH and oxygen levels of the

incubation medium were either not taken into consideration or at least not described properly. Jennings and Whitaker (1941), Nimura (1968), Jones (1972), and Metalli and Ballardin (1972) have nevertheless demonstrated that pH should not be lower than 7 in order to obtain a good hatching ; the use of unbuffered NaCl solutions may lead to a hatching efficiency which can be very low even in the controls. The toxicity of products causing a pH drop is thus overestimated. An adequate aeration of the medium is another prerequisite to obtain successful hatching, a fact underlined by Nimura (1968), Von Hentig (1971), and Sorgeloos (1975).

The major restriction, however, to use hatching as a standard bioassay criterion is the variability of the hatching efficiency and the hatching rate among different geographical strains of *Artemia* (D'Agostino, 1965 ; Sorgeloos and Persoone, 1975 ; Person-Le Ruyet and Salaun, 1977 ; Smith *et al.*, 1978) and among different batches of a specific strain (Smith *et al.*, 1978) ; these factors are also influenced by the storage conditions of the cysts (Sorgeloos, 1979).

The use of nauplii for toxicity studies

Since the publication of Corner and Sparrow (1956), *Artemia* nauplii have become a popular test object to study the toxicity of a wide array of chemical compounds : pesticides (Tarpley, 1958 ; Ludemann and Neumann, 1961 ; Springer and Legge, 1966 ; Caserio *et al.*, 1970 ; Nelson and Matsumura, 1975a ; Claus, 1976), petrochemicals and oil dispersants (Tarzwell, 1969 ; Anonymous 1973 ; Zillioux *et al.*, 1973 ; Price *et al.*, 1974), heavy metal salts (Corner and Sparrow, 1957 ; Corner and Rigler, 1958 ; Herald and Dempster, 1965 ; Wisely and Blick, 1967 ; Brown and Ahsanullah, 1971 ; Saliba and Ahsanullah, 1973 ; Cox, 1974), mycotoxins and microbial and fungal metabolites (Brown *et al.*, 1968 ; Harwig and Scott, 1971 ; De Waart *et al.*, 1972 ; Reiss, 1972 ; Durackova *et al.*, 1977), carcinogenic products (Morgan and Warshawsky, 1977) etc.

The criteria which have been taken into consideration for the dose-effect relationship differ widely. In most cases the life-death criterion, which is the most easy to detect, has been used. Some authors, however, preconize more sensitive criteria such as immobilization (Robinson *et al.*, 1965 ; Morgan and Warshawsky, 1977), osmoregulation (Schmidt-Nielsen, 1974) and oxygen consumption (Hood *et al.*, 1960). Others studied the larval growth in chronic toxicity tests (Brown and Ahsanullah, 1971 ; Saliba and Ahsanullah, 1973 ; Benijts and Versichele, 1975 ; Saliba and Krzyz, 1976b).

In most cases very little emphasis was given nor attention paid to the hatching conditions. In Cox (1974), Curtis *et al.* (1974), Granade *et al.* (1976) and Hudson and Bagshaw (1978) no details can be found on the hatching conditions. Several authors are very vague in indicating the hatching temperature, using terms such as "about" or "approximately", whereas precisely this factor influences, to a very large extent, both the hatching rate of the cysts and the molting rate of the nauplii (Sorgeloos *et al.*, 1978).

The importance of starting the tests with nauplii of exactly the same age has already been pointed out by Tarzwell (1969). With regard to this, Sorgeloos (1975), Claus (1976), and Sorgeloos *et al.* (1978) demonstrated that instar II larvae are more sensitive than instar I larvae. As a result, the data obtained by all those who do not follow a very strict procedure with regard to the hatching conditions (and thus do not know the exact age of the nauplii at the start of the tests) can fluctuate from one experiment to another.

Sorgeloos *et al.* (1978) did not observe significant differences in sensitivity between instar II and instar III nauplii and Tarpley (1958) found no significant differences among the instar III – V – VII – IX and XI stages.

As pointed out above, another factor responsible for differences in dose-effect results is the geographical origin of the cysts. Sorgeloos *et al.* (1978) found different tolerance ranges for several environmental factors between some geographical *Artemia* populations. Already in 1969, Tarzwell followed by Zillioux *et al.* (1973) emphasized the importance of the selection of one specific *Artemia* strain for bioassays.

The use of adults for toxicity studies

Results of bioassays made on adult brine shrimp have been published by many scientists : Shackell (1925), Michael *et al.* (1956), Tarpley (1958), Grosch (1966, 1967, 1970, 1973), Hallopeau (1969), Brown and Ahsanullah (1971), Knauf and Schulze (1973), Trieff *et al.* (1973), Cunningham (1976a,b), Knöfel (1976), and Saliba and Krzyz (1976b).

The following dose-effect criteria have been taken into consideration by these authors : death, life span, fecundity, reproduction capacity, survival of zygotes, and accumulation of the toxicant.

Adult *Artemia* are, however, less frequently used for toxicity studies than nauplii. Because the use of adults implies the culturing of the organisms, implying technological and biological difficulties. As a consequence, adults are less suited for short term standard toxicity tests than nauplii not the least from the economical point of view. Moreover Tarpley (1958), Brown and Ahsanullah (1971) and Saliba and Krzyz (1976b) found that the adults are less sensitive than the nauplii. Literature data revealed, however, that adult *Artemia* are very well suited for long term chronic bioassays.

Synthesis and conclusions

From the literature information cited above and data published on the sensitivity of *Artemia* as compared to other aquatic organisms (Doudoroff and Katz, 1953 ; Corner and Sparrow, 1956 ; Sanders and Cope, 1966 ; Wisely and Blick, 1967 ; Connor, 1972 ; Knauf and Schulze, 1973 ; Jung, 1975 ; and Claus, 1976) we made the following deductions with regard to the use of *Artemia* as a potential candidate species for a short-term standard bioassay :

1. To date *Artemia* is the only animal species available which, because of the commercial availability of cysts, can be used for bio-assays at any place in the world and at any moment without the necessity of maintenance of stocks.
2. *Artemia* is a suitable test species to evaluate the relative toxicity of a broad range of chemical compounds.
3. *Artemia* does not belong to the most sensitive species of marine organisms.
4. Hatchability of the cysts does not seem to be a very good criterion.
5. The early larval stages of *Artemia*, which can survive for a few days without feeding, are most suited for acute toxicity tests.
6. The sensitivity varies with the geographical origin and the age of the animals :
   - adults are less sensitive than nauplii
   - the first instar stage is less sensitive than the later instars (which all have approximately the same sensitivity).

7. In order to optimize the reproducibility of the results, the larvae should be hatched out under strictly controlled conditions of temperature, salinity, aeration, light, and pH.
8. The larvae must be of exactly the same age at the start of every experiment.

Research

Based on the premises outlined above we have endavored to work out a standardized test-procedure based on the use of *Artemia* nauplii.

The major biological, technological, and physicochemical parameters which could exert an influence on the variability of the results have been checked out one by one in different series of experiments.

Each time a choice was made and a decision taken at the end, to determine a precise experimental condition to be strictly observed or a procedure to be followed in order to obtain a maximal reproducibility in the final standard procedure.

BASIC METHODOLOGY

The following technical procedure for which we finally settled is the result of a lot of trial and error experimentation based to a considerable extent on the experience existing at the Artemia Reference Center with regard to hatching and culturing of brine shrimp.

Hatching of the cysts and collection of the nauplii

The cysts were always incubated under identical, strictly controlled conditions. For each test, 100 mg of cysts were put in 100 ml artificial seawater (35 ‰) in a cylindroconical tube at a temperature of 25 ± 1 °C. The seawater was prepared according to the formula of Dietrich and Kalle (in Kinne, 1971); after filtration (0.2 μm) and aeration, the pH was 7.5.

The cysts and hatching nauplii were kept in suspension by a gentle aeration from the bottom of the tube. Since light intensity has an influence on the hatching rate (Vanhaecke *et al.*, in preparation) the hatching tubes were placed nearby a light source (intensity of 1 000 lux).

To harvest the hatched nauplii, the aeration was stopped and the nauplii, which concentrate at the bottom of the tube, were sucked out by pipeting and transferred to a vial containing fresh seawater.

Preparation of the tests

All the experiments were carried out in glass petri dishes (diameter 60 mm ; height 12 mm). Petri dishes indeed proved to be very handy to check the mortality under a dissection microscope. The nauplii were transferred to the petri dishes with a Pasteur pipet which carried over less than 0.05 ml seawater into the dishes. The dishes were then filled with 10 ml of the respective concentrations of toxicant in seawater. Then they were closed and placed in darkness in an incubation chamber (temperature of 25 ± 1 °C) for the respective test periods. The animals were not fed during the bioassays. The concentrations of toxicant to be tested were chosen from the logarithmic scale of Doudoroff *et al.* (1951).

Preliminary trials revealed that bioassays in triplicate with 10 nauplii per petri dish appeared to be a good compromise between the precision of the test and economic imperatives such as labor and time.

At the end of the experimental period the number of dead larvae was checked in each petri dish. The nauplii were considered dead when no moving of the appendages was observed within a few seconds.

The LC50, the 95% confidence limits, and the slope function were calculated following Litchfield and Wilcoxon (1949). We also used the method developed by these authors for the statistical comparison of the LC50 values obtained. Mean values of replicates were compared at the aid of a Student's t-test.

Experiments

Selection of the instar stage of the nauplii and determination of the duration of the test

From the literature review outlined above it is clear that the nauplii must have exactly the same age at the start of every experiment because there is a marked difference in sensitivity between instar I and instar II-III larvae. From the practical point of view of handling and reproducibility it is, however, not known which stage of development is the most suited for short-term standard toxicity tests. The only information found in this regard was that by Wisely and Blick (1967), who noted that the LC50 values obtained are relatively more constant using older larvae (20-80 hr).

We therefore decided to compare both stages of development from the viewpoint of sensitivity and of practicality.

Artemia cysts from the San Francisco Bay Brand Company (batch number 1628) were incubated under the conditions outlined above. In order to obtain a population consisting of first instar nauplii only, the hatched larvae were harvested after 18 hr (the first larvae appeared 14 hr after the start of the incubation). One half of the population was used immediately for the test on instar I larvae. The other half of the population was incubated at 25 °C in an Erlenmeyer in gently aerated seawater ; regular checks of the morphological stage of development showed that after 24 hr, more than 99% of the nauplii had molted into the instar II or even instar III stage. The larvae were then collected to carry out bioassays on *Artemia* nauplii which are in the instar II-III stage.

With regard to the test period we settled in both series for 6 hr and 24 hr. The 6 hr period was selected because during that period of time instar I larvae do not yet molt.

Sodium lauryl sulphate was chosen as toxicant. After determination of the critical range in a preliminary experiment, the following concentrations were selected for further experimentation :

- instar I larvae : 10 – 13.5 – 18 – 24 – 32 – 42 – 56 – 75 – 87 – 100 mg/l :
- instar II larvae : 5.6 – 10 – 13.5 – 18 – 24 – 32 – 42 – 56 – 75 – 100 mg/l.

All bioassays were carried out in eight replicates.

In Table I the means of the LC50 values, their 95% confidence limits, and the slope function (S) are summarized. This Table also gives the standard deviation and the coefficient of variation of the LC50 values and the standard deviation of the slope function. The mortality curves computed from the mean LC50 values and the mean S values of the replicates are represented graphically in Fig. 1.

TABLE I

Mean LC 50 values, (with standard deviation and variation coefficient), slope function (with standard deviation), and 95% confidence limits of the LC 50 data obtained with instar I, respectively instar II-III larvae for 6 hr and 24 hr exposure to sodium lauryl sulphate

	Instar I		Instar II-III	
	6 hr	24 hr	6 hr	24 hr
Mean LC50 (ppm)	77.4	33.9	44.2	17.8
Standard deviation (ppm)	5.9	5.2	4.4	0.9
Variation coefficient (%)	7.7	15.3	10.0	5.1
Slope function (S)	1.16	1.23	1.31	1.35
Standard deviation	0.05	0.04	0.05	0.06
95% confidence limits of LC50's (ppm)	73.6-81.8	30.8-37.4	39.9-48.9	15.9-20.0

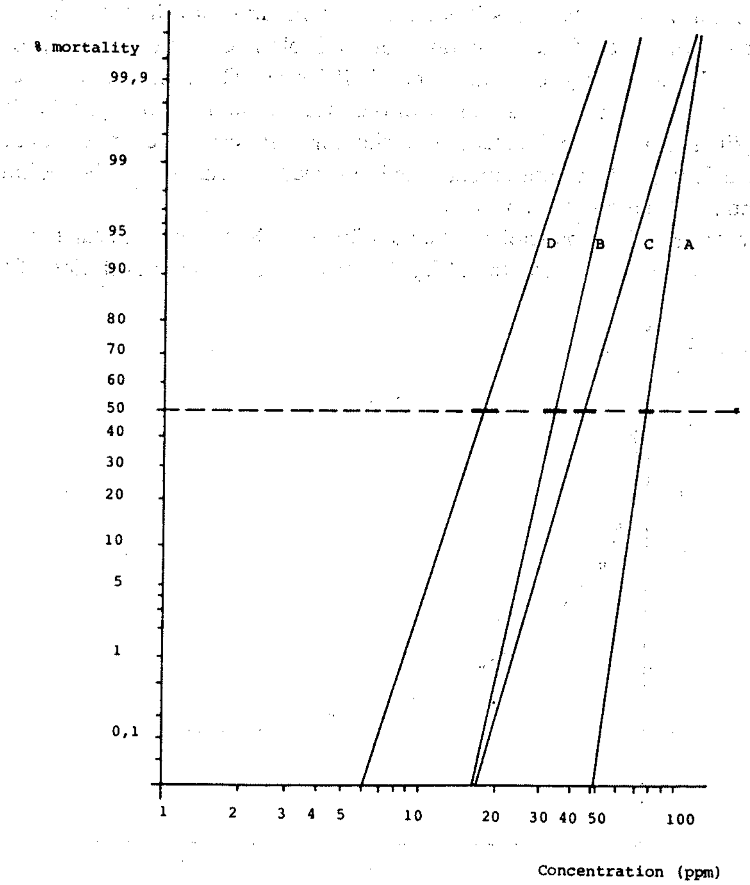

FIG. 1. Mortality curves calculated from the mean LC50's (6 hr and 24 hr) and their slope function, for instar I and instar II and III larvae respectively. The 95% confidence limits of the mean LC50's are also shown. (A) instar I larvae – 6 hr test ; (B) instar I larvae – 24 hr test ; (C) instar II-III larvae – 6 hr test ; (D) instar II-III larvae – 24 hr test.

From the results it is clear that instar II-III larvae are significantly more sensitive than instar I larvae : the ratios are 1.75 for 6 hr incubation and 1.90 for 24 hr respectively. These results confirm the findings of Wisely and Blick (1967), Claus (1976) and Sorgeloos *et al.* (1978).

In both cases, the value of the slope function for instar I larvae appears to be significantly different (at the $P < 0.01$ level) from the S values obtained for instar II-III larvae. The slope function for the 6 hr test with instar I nauplii also differs significantly from the value for the 24 hr test with these larvae. Since the S value varies with the mode of action of the toxicant (Bliss, 1957), the differences observed reveal that this mode of action is not the same for the instar I and instar II-III larvae. The intermediate value obtained for the 24 hr tests on instar I nauplii can be explained by the fact that the nauplii molted into the instar II stage during the test. An explanation for these findings can be found in the work of Sorgeloos *et al.* (1978); these authors impute the difference in sensitivity between instar I and instar II-III nauplii to the fact that in freshly hatched nauplii the epithelium of the digestive tract is not in contact with the external medium, whereas it is for the second and third instar stages.

A comparison of the coefficients of variation in Table I reveals that the reproducibility is highest for the 24 hr toxicity test with instar II-III larvae. The 6 hr test with instar I larvae comes in the second place. The variability of the LC50 value is highest for the 24 hr test on instar I nauplii. This is due to the fact that during this test period the larvae molt to the more sensitive instar II stage. As a consequence the intraspecific variability of the molting rate adds a supplementary variation to the results.

The lower degree of reproducibility obtained for the 6 hr test with instar II-III nauplii can be explained with the dose-effect curve (Fig. 2). This curve gives the evolution of the LC50 in

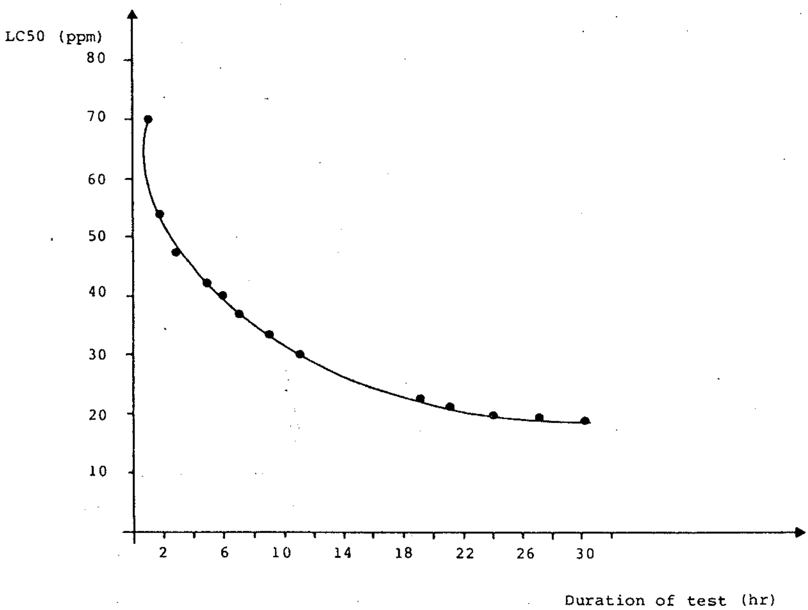

FIG. 2. Dose-effect curve for bioassays on instar II-III nauplii of *Artemia* with sodium lauryl sulphate as toxicant.

function of the duration of the test (Sprague, 1969). From Fig. 2 it is clear that after 6 hr the horizontal part of the dose-effect curve is not yet reached. As a result the 6 hr LC50 values are liable to larger fluctuations than the 24 hr LC50 values.

Prolongation of the test period beyond 24 hr does not make sense because the unfed test animals start to die in the controls. In Fig. 3 the procentual mortality of the nauplii in the controls is given in function of the length of the test period. From this figure it is clear that the mortality is already 36% 72 hr after the harvest of the nauplii. Therefore it is impossible to carry out 48 hr toxicity tests with instar II-III nauplii, except by complicating the test by feeding the animals.

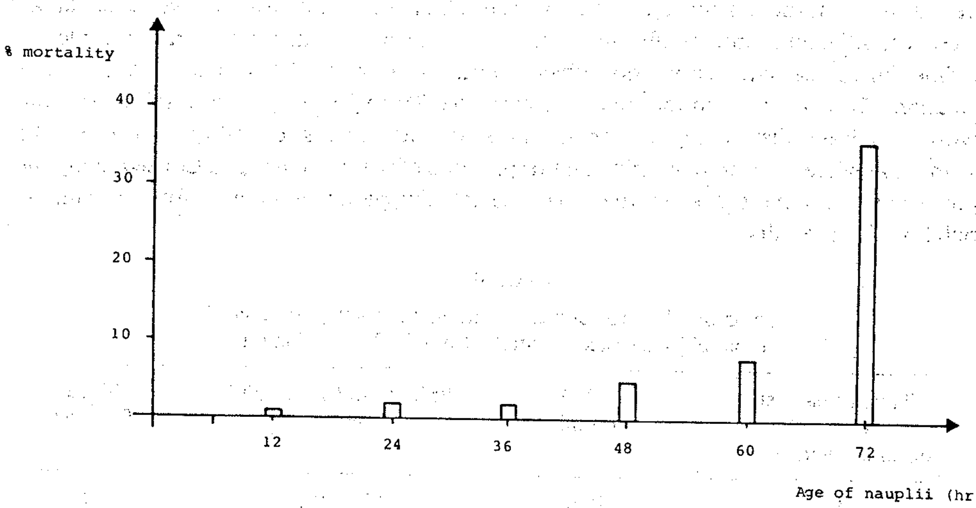

FIG. 3. Cumulative mortality of nauplii in control as a function of time.

Sensitivity of early versus late hatching nauplii
and influence of the age of the instar II-III nauplii at the start of the test

Hatching of a batch of cysts incubated under controlled conditions is not synchronous, but extends over a period of time, the length of which is related to the temperature of the hatching medium. As a result of this variability within the same batch of cysts, fast hatching nauplii appear several hours before the last nauplii hatch out.

It is therefore possible to consider that the amount of energy present in the freshly hatched larvae differs between "fast and slow" or early- and late-hatching nauplii and may influence their respective sensitivity. For this reason we decided to compare the sensitivity of nauplii hatched out at different moments after incubation and to check at the same time the influence of the age of instar II-III nauplii at the start of the test with regard to their sensitivity.

Cysts from the San Francisco Bay Brand Company (batch number 1628) were used. Sixteen hours after incubation, the hatched nauplii were separated from the unhatched cysts with a separator box (Persoone and Sorgeloos, 1972). At that moment about one third of the cysts had hatched. Two hours later a part of the embryo's in the umbrella stage were sampled and again 2 hr later the nauplii hatched out of these embryo's were collected. Twenty two hours

after incubation, when almost all cysts had hatched, one third of the remaining naupliar population was removed from the hatching tube. The last two samples of nauplii were collected 26 and 30 hr after incubation respectively.

The nauplii sampled at different moments were all incubated for exactly 24 hr at 25 °C, after which bioassays were started with sodium lauryl sulphate as toxicant. The experimental test period was 24 hr. The concentrations tested out were : 5.6 – 10 – 13.5 – 18 – 24 – 32 – 42 mg/l.

From the results (Table II) it is clear that there is no difference in sensitivity between larvae that hatch out first and those that hatch out later. A statistical comparison of the data furthermore revealed that there is no significant difference (at the $P < 0.05$ level) between the larvae harvested at different moments after incubation. This means that the larvae can be sampled at any time during the hatching period (which ranges from 14 to 24 hr) to constitute the test population. The data obtained for the larvae harvested 26, respectively 30 hr after incubation, however, indicate that a slight increase in sensitivity occurs probably related to the consumption of energy (for swimming and respiration) of the unfed nauplii. Consequently the nauplii should be harvested before the end of the hatching period in order to obtain a minimal variability of the results.

TABLE II

Results of 24 hr toxicity tests with sodium lauryl suphate on nauplii harvested at different times after incubation

Time of harvest of nauplii (hr after incubation)	LC50 (ppm)	95% confidence limits (ppm)	Slope function
16	19.0	17.6-20.6	1.29
20	19.0	17.6-20.5	1.27
22	18.8	17.2-20.5	1.33
26	17.5	16.3-18.8	1.18
30	17.4	15.9-19.0	1.20

Influence of the storage conditions of the cysts

Rakowicz (1975) noted that the viability of *Artemia* cysts decreases upon storage under humid conditions and Sorgeloos (1979) demonstrated that the hatching efficiency varies with different storage conditions. These findings indicate that biochemical changes within the embryo can occur depending upon specific storage conditions.

In order to find out whether the sensitivity of the nauplii is influenced by storage time and storage conditions of the cysts, toxicity tests were performed on instar II-III nauplii hatched out from cysts stored under air, oxygen, vacuum, nitrogen, and in brine respectively. The cysts originated from salt ponds in the San Francisco Bay and the San Pablo Bay (San Francisco Bay Brand Company, batch numbers 288-2596 and 1628 respectively).

In each case the nauplii were harvested 20 hr after incubation ; 24 hr later the bioassays were started. Sodium lauryl sulphate was used as toxicant ; the test duration was 24 hr.

TABLE III

Influence of storage period and storage conditions on sensivity of *Artemia* nauplii (24 hr-LC50 with sodium lauryl sulphate)

Storage conditions	LC50 (ppm)	95% confidence limits (ppm)
a) San Francisco Bay strain (batch 288-2596) storage period : 1 year		
Vacuum	18.5	16.8-20.4
Air	20.0	18.3-21.9
O_2	20.4	18.7-22.2
N_2	19.5	18.2-20.9
Brine	18.7	17.1-20.4
b) San Pablo Bay strain (batch 1628) storage period : 6 months		
Vacuum	17.0	15.4-18.8
Air	16.5	14.9-18.3

The data obtained are summarized in Table III. No significant differences could be observed between the LC50 values obtained. This indicates that the storage method does not affect the sensitivity of the nauplii. A comparison of the data from Table I and the results given in Table III furthermore reveals that the sensitivity of the San Francisco Bay Brand sample 1628 sample did not change due to storage over a 6 months period.

Comparative study of the sensitivity of some geographical populations and batches of Artemia

The following geographical populations and batches were studied :

- San Francisco Bay (San Francisco Bay Brand Company), USA
  - batch number 288-2596
  - batch number 288-2606
- San Pablo Bay (San Francisco Bay Brand Company – batch number 1628), USA
- Great Salt Lake (Sanders Brine Shrimp Company), USA
  - harvest 1966
  - harvest 1977
- Macau (Cirne Brand), Brazil
  - harvest May 1978
  - batch number 87500
  - batch number 871172
- Shark Bay (World Ocean – batch number 114), Australia
- Buenos Aires (Aquarium Products – harvest 1977), Argentina
- Margherita di Savoia (harvest 1977), Italy.

All samples were incubated under the standard conditions outlined above (see basic methodology). Since the various populations and batches have different hatching rates, the freshly-hatched nauplii were separated from the unhatched cysts and hatching debris when a 50% hatching success was reached.

The nauplii were then incubated for 24 hr at 25 °C. After this period, in all the populations studied, more than 99% of the larvae had molted to the instar II-III stage except the Shark Bay strain, which still contained 4-5% instar I nauplii.

Sodium lauryl sulphate and potassium dichromate were used as toxicants. The concentrations tested out were 5.6 – 10 – 13.5 – 18 – 24 – 32 – 42 – 56 mg/l for sodium lauryl sulphate and 5.6 – 10 – 18 – 32 – 56 – 100 ppm for potassium dichromate.

The results of the 24 hr toxicity tests are summarized in Tables IV and V. Fig. 4 gives the statistical comparison (at the $P < 0.05$ level) of the LC50 values obtained : from the data it is clear that there are significant differences between the geographical strains studied.

TABLE IV

24 hr-LC50 with 95% confidence limits and slope function of instar II-III larvae from different geographical strains or batches of *Artemia*, with sodium lauryl sulphate as toxicant

Origin of sample	LC50 (ppm)	95% confidence limits (ppm)	Slope function
San Pablo Bay 1628	16.0	14.9-17.2	1.27
San Pablo Bay 1628	16.7	15.6-17.9	1.25
San Francisco Bay 288-2596	16.5	15.4-17.7	1.26
San Francisco Bay 288-2606	15.9	14.4-17.6	1.26
Macau May 1978	14.8	13.7-16.0	1.29
Macau 87500	15.5	14.0-17.1	1.38
Macau 871172	15.8	14.1-17.7	1.45
Shark Bay	21.2	19.7-22.8	1.18
Buenos Aires	(13.0)*		
Margherita di Savoia	22.7	20.0-25.7	1.50
Great Salt Lake 1966	24.8	23.1-26.7	1.18
Great Salt Lake 1977	23.8	22.7-24.9	1.11

* Mortality in control : 30%.

TABLE V

24 hr-LC50 with 95% confidence limits and slope function of instar II-III larvae from different geographical strains or batches of *Artemia* with potassium dichromate as toxicant

Origin of sample	LC50 (ppm)	95% confidence limits (ppm)	Slope function
San Pablo Bay 1628	28	23.5-33.4	1.50
San Pablo Bay 1628	29	24.3-34.5	1.66
San Francisco Bay 288-2596	41	36.3-46.3	1.48
San Francisco Bay 288-2606	40	35.1-45.6	1.53
Macau May 1978	46	38.9-54.4	1.60
Macau 87500	48	40.8-56.5	1.45
Macau 871172	45	38.5-52.6	1.43
Shark Bay	64	56.7-72.3	1.32
Margherita di Savoia	58	48.3-69.7	1.52
Great Salt Lake 1966	35	30.3-40.4	1.39
Great Salt Lake 1977	32.5	27.4-38.6	1.48

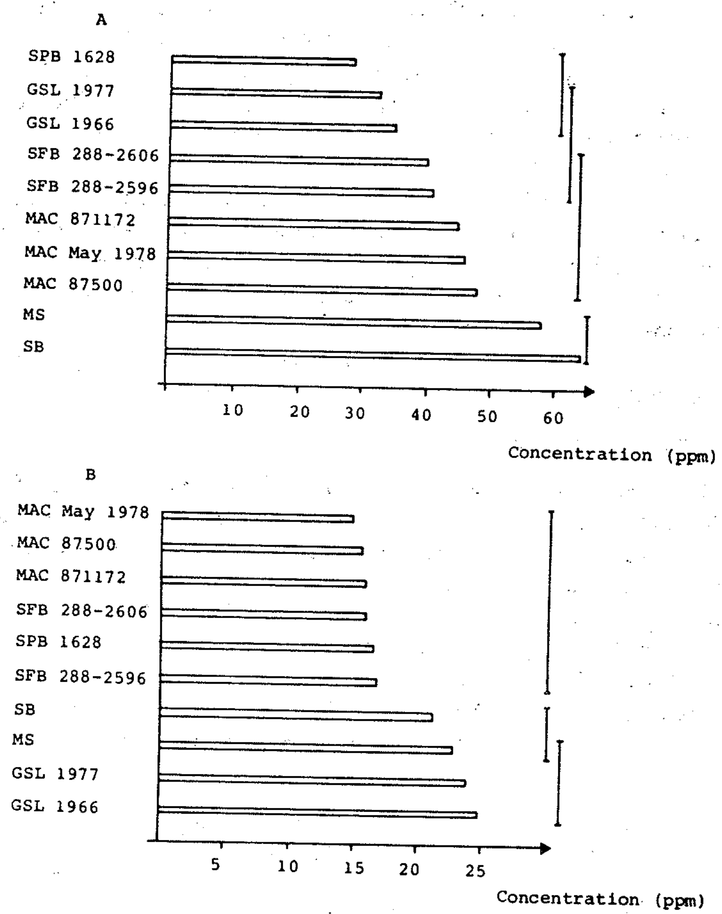

FIG. 4. 24 hr LC50 values of instar II-III larvae of different strains or batches of *Artemia* for potassium dichromate (A) and sodium lauryl sulphate (B), and statistical analysis of differences in sensitivity ($P < 0.05$ level). Any strain or batch connected by the same line was not statistically different. SFB = San Francisco Bay ; SPB = San Pablo Bay ; MAC = Macau ; MS = Margherita di Savoia ; SB = Shark Bay ; GSL = Great Salt Lake.

We have to be aware of the fact, however, that the strain with the lowest LC50 value is not necessarily the most sensitive one. Sorgeloos *et al.* (1976) and Claus *et al.* (1977) indeed indicated that the temperature-salinity tolerances vary between geographical populations. As a consequence, the LC50 values obtained are only relevant for the specific test conditions used here. The temperature-salinity combination defined and adopted earlier when working exclusively with the San Francisco Bay strain is for instance not suited for the race from Buenos Aires, for which a 30% mortality was noted in the control !

The data obtained also show that the sequence of sensitivity of different strains varies according to the product tested. This shifting points to physiological differences between these populations. It is known for example that there is an important genetic difference between the

Margherita di Savoia and the Great Salt Lake strain (Abreu-Grobois and Beardmore, 1980). This seems, however, not to be the case for the populations from San Francisco Bay and Great Salt Lake which both belong to the species *Artemia franciscana* (Bowen and Sterling, 1978), although on the other hand biometrical, chemical and physiological differences have been demonstrated between these two geographical races (Olney *et al.*, 1980 ; Schauer *et al.*, 1980 ; Vanhaecke and Sorgeloos, 1980).

In contrast to the differences found among the various geographical populations, different batches of the same geographical strain seem to give identical dose-effect results. The only exception to this rule is batch number 1628 from the San Francisco Bay Brand Company which differs significantly from the other batches from the same Company studied for $K_2Cr_2O_7$. Sorgeloos (1980) indicated that the 1628 batch labeled San Francisco Bay, originates in fact from the San Pablo Bay and should thus be considered as a different geographical population.

Selection of a standard reference toxicant

In order to check the stability of the sensitivity of the test-animals and the conformity of the experimental test procedure it was necessary to select and adopt one reference toxicant.

We decided to compare potassium dichromate, widely used as reference toxicant in aquatic toxicology with sodium lauryl sulphate $(CH_3)(CH_2)_{10}CH_2OSO_3Na$ already recommended for *Artemia* by several authors (Tarzwell, 1969 ; Anonymous, 1973 ; Zillioux *et al.*, 1973 ; Price *et al.*, 1974). A series of nine replicate 24 hr bioassays were carried out on instar II-III nauplii from San Pablo Bay (batch number 1628) with these two chemicals.

The results are summarized in Tables VI and VII. The mean mortality curve and the mean 95% confidence limits for both products are represented graphically in Fig. 5.

TABLE VI

Results of a toxicity test in nine replicates on instar II-III nauplii exposed to sodium lauryl sulphate (24 hr LC50)

Statistics	LC50 (ppm)	95% confidence limits (ppm)	Slope function
	15.8	14.5-17.2	1.32
	15.5	14.3-16.8	1.29
	18.5	17.1-20.1	1.30
	18.4	16.7-20.3	1.47
	18.1	16.7-19.6	1.38
	15.6	14.3-17.0	1.33
	19.0	17.7-20.4	1.26
	18.8	17.2-20.5	1.33
	17.0	15.6-18.5	1.32
$\bar{X}$	17.4	16.0-18.9	1.33
s	1.45		0.06
%s	8.3		4.6

From the LC50 values it is clear that *Artemia* larvae are more sensitive to sodium lauryl sulphate than to potassium dichromate. A comparison of the two slope functions furthermore

TABLE VII

Results of a toxicity test in nine replicates on instar II-III nauplii exposed to potassium dichromate (24 hr-LC50)

Statistics	LC50 (ppm)	96% confidence limits (ppm)	Slope function
	27.9	23.5-33.1	1.73
	30.0	24.6-36.5	1.89
	36.0	29.6-43.8	2.18
	28.1	23.1-34.2	1.88
	34.5	29.5-40.3	1.65
	33.0	27.8-39.2	1.97
	39.0	33.2-45.9	1.69
	28.0	23.5-33.4	1.50
	31.0	26.0-36.9	1.76
$\bar{X}$	31.9	26.6-28.3	1.81
s	3.96		0.20
%s	12.4		11.0

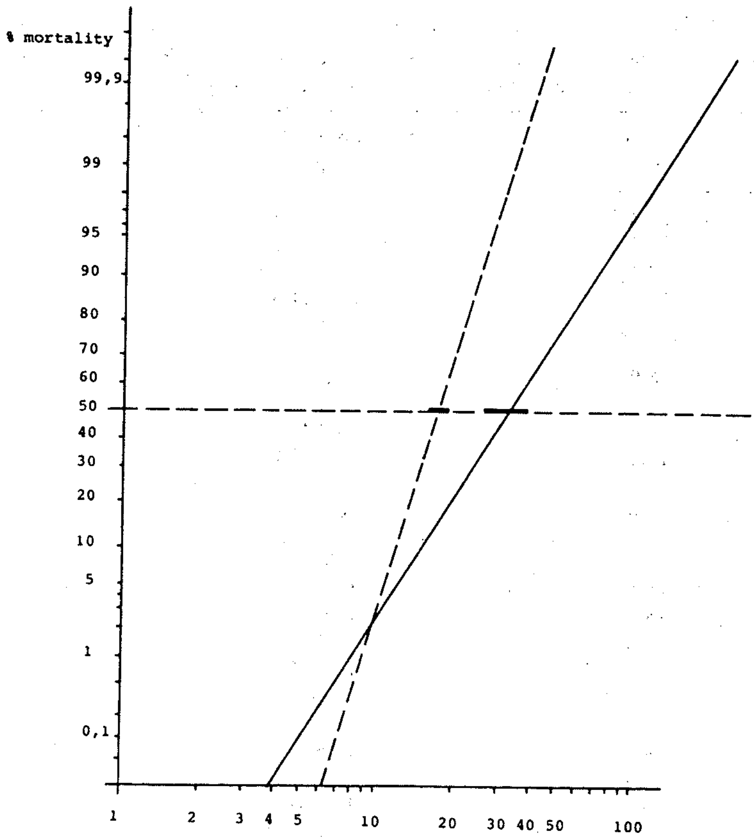

FIG. 5. Mortality curves calculated from the mean 24 hr LC50's and their slope function for instar II-III larvae, exposed to sodium lauryl sulphate (– –) and potassium dichromate (—). The 95% confidence limits of the mean LC50's are also shown.

indicates a different mode of action for both products. Sodium lauryl sulphate acts within a narrower range of concentrations. As a result the 95% confidence interval with this product is closer to the LC50 ($\pm$ 9%) than it is for $K_2 Cr_2 O_7$ ($\pm$ 20%). Moreover, a comparison of the variation coëfficients shows that the reproducibility of the bioassays is much better with sodium lauryl sulphate. On the basis of these results we decided to advise sodium lauryl sulphate as reference toxicant.

Determination of the accuracy and repeatability of the test ;
establishment of the acceptable limits of the LC50 range for sodium lauryl sulphate

A 24 hr. toxicity test has been carried out in 25 replicates on instar II-III larvae from the geographical population of Macau (harvest May 1978). For each replicate a new cyst sample was incubated and a freshly made solution of sodium lauryl sulphate used. The following concentrations of the reference toxicant were tested : 10 – 13.5 – 18 – 24 – 32 mg/l.

The LC50 values of the 25 replicates, the 95% confidence limits and the S values are summarized in Table VIII. The mean mortality curve is represented in Fig. 6. The 95%

TABLE VIII

Results of a toxicity test in 25 replicates on instar II-III larvae from Macau cysts exposed to sodium lauryl sulphate (24 hr LC50)

Statistics	LC50 (ppm)	95% confidence limits (ppm)	Slope function
	18.0	16.4-19.7	1.37
	16.2	14.4-18.3	1.40
	19.4	17.6-21.4	1.32
	19.6	17.9-21.4	1.28
	18.0	16.2-20.0	1.43
	17.6	15.3-20.3	1.35
	16.9	15.3-18.7	1.33
	15.6	13.6-17.9	1.46
	16.1	14.8-17.5	1.27
	15.5	14.2-17.0	1.29
	16.1	14.5-17.9	1.35
	18.0	16.2-19.9	1.42
	15.7	14.0-17.6	1.37
	16.1	14.0-18.6	1.32
	15.6	13.4-18.1	1.26
	15.2	13.0-17.8	1.25
	16.6	15.2-18.1	1.28
	16.2	14.2-18.5	1.45
	15.9	14.4-17.5	1.31
	16.3	14.6-18.2	1.37
	14.5	12.9-16.3	1.38
	16.9	14.6-19.5	1.50
	16.7	15.0-18.6	1.35
	15.8	14.5-17.2	1.27
	15.7	14.3-17.2	1.29
$\bar{X}$	16.6	14.8-18.5	1.35
s	1.25		0.07
%s	7.5		5.1

confidence limits are within 11 % of the mean LC50 value. The extreme LC50 values recorded are 87 % and 118 % respectively of the mean LC50.

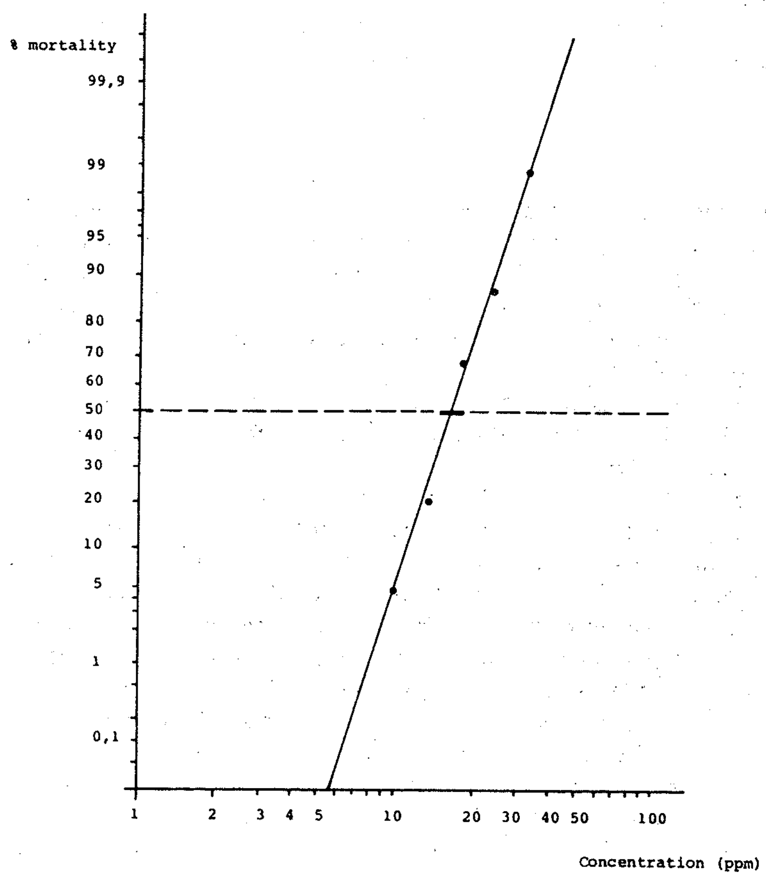

FIG. 6. Mortality curve calculated from the mean 24 hr LC50 and the slope function of 25 replicate tests on instar II-III larvae with sodium lauryl sulphate as toxicant. The 95 % confidence limits of the mean LC50 is also shown.

Two questions have to be answered : are these deviations acceptable from the statistical point of view and what is the maximum range of variation which can be accepted for a standardized test with a reasonable accuracy. For this purpose it was necessary to calculate the minimum procentual deviation which an LC50 may have from the mean LC50 value obtained (16.6) to differ significantly at the 0.05 level. The method of Litchfield and Wilcoxon (1949) was used to compare this mean LC50 value with a hypothetical LC50 derived from a mortality curve with a slope function varying between 1.35 ± 2 s (s = standard deviation of S). This calculation revealed that a ratio $\frac{\text{LC50 m}}{\text{LC50 h}} = 1.195$ (with LC50m = 16.6 and LC50h = the

hypothetical LC50) is necessary in order to obtain a significant difference at the $P < 0.05$ level between the hypothetical LC50 value and the mean. All LC50 values within a range of 20 % of the mean LC50 value are thus acceptable.

In practice this means that the interval in which the LC50 value of the reference toxicant must be situated, ranges from 13.3 to 19.9 ppm.

Conclusions

From the literature it is clear that brine shrimp are very well suited for the predictive assessment of the toxicity of a wide variety of compounds. However, as demonstrated above, none of the methods proposed so far, is acceptable as a standard procedure for a simple, routine, short-term toxicity test either because of the lack of precision in the description of the experimental procedure or because some basic biological characteristics of this particular test-species had been overlooked.

Starting from the abundant literature information a number of experiments were carried out in which the influence of several factors on the repeatibility of the results and the practicality of the test procedure were carefully checked.

A 24 hr toxicity test of nauplii which are all in the instar II-III stage was found to be best suited for a simple routine test. Not only are instar II-III nauplii more sensitive than instar I nauplii and adults, but it appeared to be more difficult to work out a good experimental procedure to obtain a homogenous instar I population than a mixed population of instar II and III larvae. The nauplii may be harvested at any time during the hatching process to constitute the test population ; no difference in sensitivity could indeed be observed between fast and slow hatching larvae. A homogenous instar II-III population is obtained easily by a 24 hr storage of freshly hatched nauplii at 25 °C. As far as the duration of the bioassay is concerned, a 24 hr toxicity test with instar II-III nauplii gives results with the highest reproducibility. Longer test periods must be rejected because the test animals die off due to starvation. Feeding would not only complicate the test procedure, but could bias the results by the adsorption of the toxicant on the food and its subsequent ingestion by the test species.

Since it was demonstrated that different populations have different ecological optima and different sensitivities, one geographical strain had to be selected for the standard bioassay. Presently, the geographical population from the salt ponds of Macau (Brazil) seems to be the most suited. The Brazilian cysts are indeed commercially available worldwide and the exact origin of these cysts is guaranteed. The pesticide and heavy metal level of this strain is much lower than that of some other populations (Olney *et al.*, 1980) and no differences in sensitivity could be observed between various batches of this population.

The major advantage of *Artemia* for toxicity studies is the overall availability of the dry cysts ; one and the same sample of cysts can be used for at least 1 year. The sensitivity of the nauplii is not affected by storage of the cysts under different conditions. Storage under dry conditions is, however, highly recommended to maintain a high degree of hatching.

From the comparison of the data for repeatability of bioassays with *Daphnia* and *Brachydanio* (Cabridenc, 1979) and the values which we obtained for *Artemia* nauplii (Tables VI, VII and VIII) it is clear that the reproducibility of this standard *Artemia* test equals at least that of the internationally adopted tests on *Daphnia* and *Brachydanio*.

A standard short-term test with *Artemia* nauplii is attractive for many reasons. It does not require elaborate equipment nor much labor and time. The preparations to hatch the cysts are made in a few minutes, the pipeting of the nauplii into the petri dishes takes, with a little skill, not more than 30 sec per dish and the counting of the dead nauplii takes to the most a quarter of an hour per experiment. As a result it can be considered as a very handy and certainly one of the cheapest toxicity tests ever proposed.

Persoone (1980) reviewed the factors considered to be important in the elaboration of routine aquatic eco-toxicological tests, pointing to the basic antagonism between the ecological representativeness and sensitivity on one hand, and manageability, practicality, and costs on the otherhand.

It is clear that the *Artemia* test which we worked out also fits into this antagonism, since it is not a very sensitive test (at least with the life-death criterion used) and certainly not representative for the freshwater environment. On the other hand it is most suited for the marine and brackish-water ecosystem, including effluent testing in estuaries and dumpings at sea ; it can also be used as any other bioassay for the basic, range-finding level of the toxicity of chemical compounds.

In the latter context the simplicity and cheapness of this test must be weighed against the two biological criteria (representativeness and sensitivity).

To conclude let us mention that the detailed and precise experimental procedure to be followed and which is the result of this study has been published elsewhere (Vanhaecke *et al.*, 1980) under the title "Proposal for a standard short-term toxicity test with *Artemia* nauplii" and that it is presently used in intercalibration exercises in North-America and Europe.

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